

Amendments to the Specification

Please insert the following paragraph at page 1, line 3 of the specification:

--RELATED APPLICATIONS

This application is a national stage application, filed under 35 U.S.C. §371, of PCT/EP2005/051431, filed on March 30, 2005, which claims priority to Danish Patent Application No. PA 2004 00510, filed on March 30, 2004; to USSN 60/575,086, filed on May 28, 2004; and to Danish Patent Application No. PA 2004 00843, filed on May 28, 2004. Each of these applications is herein incorporated by reference in its entirety.--

Please insert the following Abstract on a separate page at the end of the specification:

--ABSTRACT

The present invention relates to the field of therapeutic use of proteins, genes and cells. More specifically the invention relates to therapy based on the biological function of a secreted therapeutic protein, NsG33, in particular for the treatment of disorders of the nervous system. NsG33 is a nerve survival and growth factor with antiapoptotic effects on a cell line with neuronal potential and with neuroprotective and/or neurogenesis effects on a neural precursor cell line and on primary striatal cultures. The invention also relates to novel bioactive NsG33 polypeptide fragments and the corresponding encoding DNA sequences.--

Please insert the sequence listing, pages 1-40, at the end of the specification.

Please replace page 7, line 35 through page 8, line 8 with the following:

-- Figure 3a: Clustal W (1.82) multiple sequence alignment of human (SEQ ID NO: 3), partial mouse (SEQ ID NO: 8) and rat NsG33 (SEQ ID NO: 13). The signal sequences are shown in bold. A putative furin cleavage site has been underlined.

Figure 3b: Clustal W (1.82) multiple sequence alignment of human (SEQ ID NO:3), mouse (SEQ ID NO: 26) and rat NsG33 (SEQ ID NO: 13). The predicted signal sequences are shown in bold.

- * indicates positions which have a single, fully conserved residue.
- : indicates that one of the following 'strong' groups is fully conserved:
 - STA, NEQK (SEQ ID NO: 27), NHQK (SEQ ID NO: 28), NDEQ (SEQ ID NO: 29), QHRK (SEQ ID NO: 30), MILV (SEQ ID NO: 31), MILF (SEQ ID NO: 32), HY, FYW.
- . indicates that one of the following 'weaker' groups is fully conserved:
 - CSA, ATV, SAG, STNK (SEQ ID NO: 33), STPA (SEQ ID NO: 34), SGND (SEQ ID NO: 35), SNDEQK (SEQ ID NO: 36), NDEQHK (SEQ ID NO: 37), NEQHRK (SEQ ID NO: 38), VLIM (SEQ ID NO: 39), HFY.--

Please replace page 8, lines 18 - 29 with the following:

--Figure 5: Align0 alignment of full length human NsG33 polypeptide (SEQ ID NO: 3) against full-length human polypeptide (Innog.) (SEQ ID NO: 40) from WO 93/22437 (Innogenetics SA). Scoring matrix BLOSUM50, gap penalties: -12/-2. The ten conserved cysteines are shown in bold with asterisks above or below the aligned sequences.

Figure 6: Human NsG33 cDNA (SEQ ID NO: 2) and encoded prepro-NsG33 (SEQ ID NO: 3)

Figure 7a: Partial mouse NsG33 cDNA (SEQ ID NO: 7) and encoded partial pre-pro-NsG33 (SEQ ID NO: 8)

Figure 7b: Full length mouse NsG33 cDNA (SEQ ID NO: 25) and encoded pre-pro-NsG33 (SEQ ID NO: 26)

Figure 8: Rat NsG33 cDNA (SEQ ID NO: 12) and encoded pre-pro-NsG33 (SEQ ID NO: 13).--

Please replace the paragraph at page 12, line 25 through page 13, line 2, with the following amended paragraph:

-- Human NsG33 contains an N-terminal signal peptide sequence of 23 amino acids, which is cleaved at the sequence motif ARA-GY (SEQ ID NO: 41). This signal peptide cleavage site is predicted by the SignalP method (see example 2) and the output graph shown in FIG.1. However, one of skilled in the art will recognize that the actual cleavage site may be different than predicted by the computer program. For example the signal peptide prediction in rat NsG33 results in predictions with approximately equal probabilities at position 16 and 21. A signal peptide cleavage site is found at a similar location in the mouse NsG33 (pos. 24) and rat NsG33 (pos. 16 or 21). Cleavage of the signal peptide results in polypeptides having SEQ ID No. 4, 9, and 14 for human, mouse, and rat respectively. As it is known in the art, signal peptide processing is not always exactly as predicted and actual cleavage may vary from case to case. Thus, it is expected that the N-terminal of mature NsG33 may vary by one to two or three amino acids from the predicted cleavage site. The actual N-terminal of mature NsG33 can be verified experimentally by C-terminal tagging with e.g. a his-tag, subsequent purification using a poly-his specific antibody or purification on a Ni column, and finally N-terminal sequencing of the purified mature peptide.--

Please replace the paragraph at page 13, lines 4-16 with the following amended paragraph:

--General-type proprotein cleavage is predicted in human NsG33 by the ProP method (Prediction of proprotein convertase cleavage sites. *Peter Duckert, Søren Brunak and Nikolaj Blom*. Protein Engineering, Design and Selection: 17: 107-112, 2004) at pos. 127 with a score of 0.831, sequence motif 'WGPRERR-AL' (SEQ ID NO: 42). Similar, cleavage sites are predicted in homologous positions in mouse NsG33 (at pos. 128) with a score of 0.831, sequence motif 'WGPRERR-AL' (SEQ ID NO: 42) and in rat NsG33 (at pos. 125) with a score of 0.831 and the sequence motif 'WGPRERR-AL' (SEQ ID NO: 42). A possible furin propeptide cleavage site is also found at position 121 in human NsG33 at sequence motif 'GGRCVR-WG' (SEQ ID NO: 43) and at corresponding positions in rat and mouse NsG33. Polypeptide processing after cleavage of the signal peptide results in the formation of a C-terminal peptide and an N-terminal peptide. Whether the protein is actually processed at these predicted sites may depend on the cell type, in which the gene is expressed. Propeptide cleavage can be experimentally verified by C-terminal his-tagging, purification and subsequent N-terminal sequencing of the tagged peptide.--

Please replace the paragraph at page 21, lines 16-22 with the following amended paragraph:

--~~Substitutions~~ Substitutions within the following group (Clustal W, 'strong' conservation group) are to be regarded as conservative substitutions within the meaning of the present invention

-STA, NEQK (SEQ ID NO: 27), NHQK (SEQ ID NO: 28), NDEQ (SEQ ID NO: 29), QHRK (SEQ ID NO: 30), MILV (SEQ ID NO: 31), MILF (SEQ ID NO: 32), HY, FYW.

~~Substitutions~~ Substitutions within the following group (Clustal W, 'weak' conservation group) are to be regarded as semi-conservative substitutions within the meaning of the present invention

-CSA, ATV, SAG, STNK (SEQ ID NO: 33), STPA (SEQ ID NO: 34), SGND (SEQ ID NO: 35), SNDEQK (SEQ ID NO: 36), NDEQHK (SEQ ID NO: 37), NEQHRK (SEQ ID NO: 38), VLIM (SEQ ID NO: 39), HFY.--

Please replace the paragraph at page 26, lines 28-36 with the following amended paragraph:

--In one embodiment, a variant NsG33 at corresponding positions comprises the residues marked in Figure 3a as fully conserved (*), more preferably a variant NsG33 also comprises at corresponding positions the residues marked in Figure 3a as strongly conserved (: strongly conserved groups include: STA, NEQK (SEQ ID NO: 27), NHQK (SEQ ID NO: 28), NEDQ (SEQ ID NO: 58), QHRK (SEQ ID NO: 30), MILV (SEQ ID NO: 31), MILF (SEQ ID NO: 32), HY, FYW), more preferably a variant NsG33 also comprises at corresponding positions the residues marked in Figure 3a as less conserved (. less conserved groups include: CSA, ATV, SAG, STNK (SEQ ID NO: 33), STPA (SEQ ID NO: 34), SGND (SEQ ID NO: 35), SNDEQK (SEQ ID NO: 36), NDEQHK (SEQ ID NO: 37), NEQHK (SEQ ID NO: 59), NEQHRK (SEQ ID NO: 38), VLIM (SEQ ID NO: 39), HFY). In particular, it is contemplated that the conserved cysteines (Figure 5) must be located at corresponding positions in a variant NsG33.--

Please replace the paragraph at page 79, lines 1-9 with the following amended paragraph:

--Protein processing:

Human NsG33 contains an N-terminal signal peptide sequence of 23 amino acids which is cleaved at the sequence motif ARA-GY (SEQ ID NO: 41). This signal peptide cleavage site is predicted by the SignalP method (Nielsen *et al.*, 1997) and the output graph shown in FIG.1. A signal peptide cleavage site is found at a similar location in the mouse NsG33 (pos. 24) and rat NsG33 (pos. 16 or 21). The most likely cleavage of rat NsG33 is at position 21 as this corresponds to the predicted cleavage position in both human and mouse NsG33. This means that the most likely N-terminal of SEQ ID No. 14, 21, and 24 is GYSEDRCS (SEQ ID NO: 44) and not the ASARAGYSED (SEQ ID NO: 45) shown in the sequence listing.--

Please replace the paragraph at page 79, lines 15-20 with the following amended paragraph:

--Protein processing:

General-type proprotein cleavage is predicted in human NsG33 (SEQ ID No 3) by the ProP method at pos. 127 with a score of 0.831, sequence motif 'WGPRERR-AL' (SEQ ID NO: 42). Similarly, a cleavage site is predicted in mouse NsG33 (SEQ ID No 8) at pos. 128 with a score of 0.831, sequence motif 'WGPRERR-AL' (SEQ ID NO: 42) and in rat NsG33 (SEQ ID No 13) at pos. 125 with a score of 0.831 and the sequence motif 'WGPRERR-AL' (SEQ ID NO: 42).--

Please replace page 82, lines 1-4 with the following:

--Soares, [1996], Genomics 33: 151-152) obtained from RZPD, Berlin, Germany (RZPD clone ID: IRALp962D105Q2) using the following primers:

5' primer: 5'-GCGGATCCAGCGGTGGTGAGAGCCCCGAC-3' (SEQ ID NO: 46)

3' primer: 5'-TATACTCGAGGCCACCCTCCCTCCTACCAG-3' (SEQ ID NO: 47)--

Please replace the paragraph at page 82, line 33 through page 83, line 10 with the following amended paragraph:

-- Real-time PCR was performed in an Opticon-2 thermocycler (MJ Research), using LightCycler-FastStart DNA Master SYBR Green I kit (Roche). Studies were carried out in duplicates using primers 5'-CCAGCGACTTCGTAATTCAC-3' (5' primer) (SEQ ID NO: 48) and 5'-AGCCCATGAAGAGGAAGG-3' (3' primer) (SEQ ID NO: 49). For Real-Time PCR, a standard curve was prepared by serial dilution of a gel-purified PCR product, prepared using the above primers. The standard curve was used to verify that crossing-point values (CT) of all samples were within the exponential range of the PCR reaction and to calculate final expression levels. All RT-PCR amplifications were performed in a total

volume of 10 µl containing 3 mM MgCl₂, 12 % sucrose and 1x reaction buffer included in the LightCycler kit. PCR cycling profile consisted of a 10 minutes pre-denaturation step at 98 °C and 35 three-step cycles at 98 °C for 10 seconds, at 62 °C for 20 seconds and at 72 °C for 20 seconds. Following the extension step of each cycle, a plate reading step was added (80 °C, 2 seconds) to quantify the newly formed PCR products. The specificity of the amplification reaction was determined by performing a melting curve analysis of the PCR fragments by slowly raising the temperature from 52 °C to 95 °C with continuous data acquisition. --

Please replace the paragraph at page 83, lines 12-17 with the following amended paragraph:

--For normalization purposes, all cDNAs were subjected to real-time PCR using primers for β₂-microglobulin (B2M, 5'-TGTGCTCGCGCTACTCTCTC-3' (SEQ ID NO: 50) and 5'-CTGAATGCTCCACTTTTTCAATTCT-3' (SEQ ID NO: 51). Standard curves for β₂-

microglobulin were prepared similar to NsG33. Housekeeping gene real-time PCR was done using the same kit as for the target gene, except that optimal annealing temperatures were used for the housekeeping gene.--

Please replace page 92, lines 25-32 with the following:

--The following primers were used for real-time PCR:

mNsG33:

mNsG33 intronspan bp284 5': 5'-GTCTTCGCTGAACGTATGAC -3' (SEQ ID NO: 52)

mNsG33 intronspan bp623 3': 5'-CTGATTCTTGACAGCTCTGTG -3' (SEQ ID NO: 53)

GAPDH:

mGAPDH-s904: 5'-AACAGCAACTCCCACTCTTC-3' (SEQ ID NO: 54)

mGAPDH-as1067: 5'-TGGTCCAGGGTTTCTTACTC-3' (SEQ ID NO: 55)--

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Please replace the paragraph at page 96, line 34 through page 97, line 2 with the following amended paragraph:

-- For normalization purposes, all cDNAs were subjected to real-time PCR using primers 4842 and 4843 for the housekeeping gene GAPDH [4842: 5'-GGAAGGTGAAGGTCGGAGTCAA-3' (SEQ ID NO: 56) and 4843: 5'-GATCTCGCTCCTGGAAGATGGT-3'] (SEQ ID NO: 57). Following normalization with GAPDH, relative expression levels of the target genes were calculated using cDNA samples with the lowest hNsG33 expression as a reference.--